Characterization, cloning and immunogenicity of antigens released by lung-stage larvae of *Schistosoma mansoni*

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SUMMARY

Lung-stage schistosomula are the target of protective immunity in mice vaccinated with attenuated cercariae of *Schistosoma mansoni*. Therefore, proteins present at this developmental stage, and in particular those which are secreted, are a potential source of novel vaccine candidates. However, little information is available about such molecules. Here we describe the cDNA clones identified by screening expression libraries with serum raised against proteins released by lung-stage schistosomula. In total, 11 different cDNA species were identified, 6 of which have been described previously in *S. mansoni*; these included fructose 1,6-*bis*phosphate aldolase and Sm21.7 which together accounted for two-thirds of all positive clones. Of the 5 newly described schistosome genes, 1 cDNA had a high degree of homology to the s5a subunit of 26S proteasomes, most signifcant being with the human protein. The remaining 4 clones showed no significant homologies to any genes sequenced previously. Fructose 1,6-*bis*phosphate aldolase, Sm21.7, the proteasome homologue and 1 unknown clone (A26) have been expressed in a bacterial expression system and serum produced against each recombinant protein. Immunolocalization showed fructose 1,6-*bis*phosphate aldolase, Sm21.7 and the proteasome homologue to be most abundant in muscle cells whilst clone A26 was distributed throughout many tissues, but was most abundant in the tegument. Analysis of the cellular immune responses of vaccinated mice showed 3 of the 4 expressed clones to be highly immunogenic, inducing the secretion of large quantities of the Th1-type cytokine interferon gamma.

Key words: *Schistosoma mansoni*, released proteins, sequence analysis, immunolocalization, immune response.

INTRODUCTION

It is well established that the immunization of mice with radiation-attenuated cercariae of *Schistosoma mansoni* induces consistently high levels of resistance against a subsequent challenge with normal parasites (reviewed by Coulson, 1997). Dissection of the murine immune response in this model has facilitated the identification of several parameters which are essential for the development of protection. For example, it is clear that T cells play a pivotal role in the expression of resistance in mice singly-vaccinated with attenuated cercariae (Vignali *et al*. 1989). More specifically, ablation of the Th1-associated cytokine interferon gamma (IFNγ), at the time of challenge, results in almost total abrogation of immunity (Smythies, Coulson & Wilson, 1992*a*). Despite the information which has accumulated detailing the immunological aspects of both the induction of immunity and the effector mechanism, little is known about the antigen(s) which elicit these responses. However, from the information available regarding the nature of the induction and effector mechanisms, several deductions can be made about the source of such molecules. Since attenuated parasites fail to develop beyond the lung-stage, proteins unique to the adult worm can be discounted as important inducers of immunity in this model system. In addition, it has been shown that attenuated lung-stage schistosomula administered intradermally are capable of eliciting high levels of protection against a subsequent challenge infection (Coulson & Mountford, 1989). Thus, antigens specific to the cercaria are not required for the induction of immunity. As parasite elimination occurs in the lungs and is T cell-mediated we can further conclude that it must be triggered by antigen(s) released from the live challenge schistosomulum for processing by accessory cells and subsequent presentation to pulmonary Th cells.

The pattern of proteins released by lung-stage schistosomula during *in vitro* culture has been described previously (Harrop & Wilson, 1993). Such molecules were found to be the most potent stimulators of IFNγ secretion by T cells recovered from the lymph nodes and lungs of vaccinated mice, compared to proteins derived from other life-cycle stages (Mountford, Harrop & Wilson, 1995). Since the protective immune mechanism depends upon a strong pulmonary Th1 response (Coulson, 1997) the proteins released by lung schistosomula are a potential source of vaccine candidates and, as such, worthy of more detailed analysis.

Only minute quantities of protein are released by larval parasites, making a detailed study of their function, immunogenicity and vaccine potential

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difficult. For this reason, serum was raised against such antigens and used to screen cercarial, lung-stage schistosomula and adult worm cDNA libraries. Positive clones have been purified, the nucleotide sequence determined and used to search DNA and amino acid databases to identify homologies. Several clones, selected for further study, were expressed in a bacterial high-level expression vector and the resulting purified protein used to produce antiserum. Each serum was used to immunolocalize the native protein in sections of cercariae, lung-stage schistosomula and adult worms and to probe Western blots of different parasite preparations. Such studies provide an insight into the tissue distribution and function of each molecule. In addition, the immunogenicity of each recombinant protein was assessed by measuring proliferation and cytokine production by cells recovered from vaccinated mice.

MATERIALS AND METHODS

Preparation of parasite antigens

The life-cycle of *S. mansoni* was maintained in the laboratory by routine passage through the intermediate snail host *Biomphalaria glabrata* and MF1 mice. Soluble protein preparations of cercariae (SCAP), lung-stage schistosomula (SLAP) and adult worms (SWAP) and proteins released by schistosomula cultured *in vitro* during the first 3 h and between day 6 and day 8 post-transformation were produced as described previously (Harrop & Wilson, 1993). Skin-stage (0–3 h RPs) and lung-stage (6–8 day RPs) parasite-released material was concentrated by centrifugation in Ultrafree centrifugal filter devices (Millipore) containing 5 kDa cut-off membranes, and the protein concentration estimated subsequently by the Bradford assay. In addition, lung worms ranging from 6 to 8 days old were frozen at −70 °C until required as a source of mRNA.

Generation of a lung-stage schistosomula cDNA library

It is not known whether the protective antigens which mediate lung-phase immunity are stage specific or are expressed throughout the parasite life-cycle. In order to maximize the chances that the relevant molecular species were present, mRNA was extracted using the MicroFast track kit (Invitrogen) from approximately 3×10^6 schistosomula which had been cultured *in vitro* for 6, 7 or 8 days (i.e. to the lung stage of development). The mRNA was subsequently packaged into the λZAP II vector by Stratagene. Two additional libraries were used throughout the course of these studies, a cercarial and an adult worm cDNA library which were kindly donated by Dr E. Dias Neto and Dr R. Pierce.

Production of antisera to schistosomula-released proteins and recombinant proteins

Antisera against 6–8 day RPs were produced by immunizing rabbits subcutaneously with approximately 20 μg of the heterogeneous antigen preparation emulsified in Complete Freund's adjuvant for the primary inoculation and in Incomplete Freund's adjuvant for the 2 subsequent boosts. The antibody titre against 6–8 day RPs was assessed by ELISA (data not shown) and deemed to be sufficient after 2 boosts. Antisera against purified recombinant proteins were produced in the same manner with the exception that mice with a disrupted IFNγ receptor gene (129 IFNγR$^{-/-}$) were used. Approximately 10 μg of purified recombinant protein were administered per mouse in both primary inoculations and in subsequent boosts.

Western blotting

Proteins were separated electrophoretically through 12 % polyacrylamide gels under reducing conditions according to the method of Laemmli (1970). Either 5 μg (heterogeneous antigen preparations) or 0·5 μg (recombinant) protein was loaded per lane. Resolved fractions were electroblotted onto Immobilon-P membrane (Millipore, Watford, UK) and probing of the blots was carried out subsequently as described by Cutts & Wilson (1997).

Screening of cDNA libraries

The cDNA libraries were screened essentially as described by Huynh, Young & Davis (1986). Nitrocellulose filters (Sartorius), were probed with primary antiserum diluted to 1:200 and positive clones visualized following staining with DAB. Recombinant bacteriophage were isolated from the test plate and purified to homogeneity by several rounds of re-screening.

Plasmid DNA preparation and sequence analysis

cDNA inserts from positive clones were subcloned into pBluescript using the Helper phage R408 (Stratagene) according to the manufacturer's instructions. Subsequently, plasmid DNA was isolated using a plasmid purification kit (Qiagen) and the purity and yield quantified spectrophometrically.

Purified plasmids were sequenced using a Dye Deoxy terminator cycle sequencing kit (ABI) according to the manufacturer's recommendations. Products were electrophoresed and analysed on an Applied Biosystems Model 373A DNA sequencing system. The resulting cDNA sequence was edited and any homology to previously sequenced genes determined by searching the GenBank and

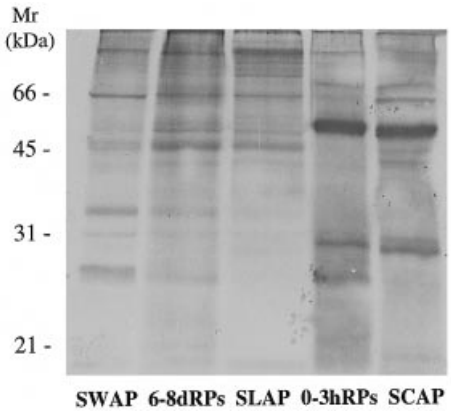

Fig. 1. Western blot of SCAP, 0–3 h RPs, SLAP, 6–8 day RPs and SWAP probed with antiserum raised against 6–8 day RPs (diluted to 1:100). 5 μg of each protein was electrophoresed on 12% acrylamide gels. Molecular weights are illustrated.

SwissProt databases using software developed by the Genetics Computer Group, University of Wisconsin, Madison, WI. A number of cDNA clones were sequenced in their entirety. In such cases, primer walking from both 5′ and 3′ ends was undertaken and the resulting sequences assembled into contigs using the Fragment Assembly System (GCG).

High-level expression

A number of clones were selected for expression in a prokaryotic high-level expression system (pQe; Qiagen). Suitable restriction sites were chosen and cDNA inserts digested out of the recombinant pBluescript and subsequently ligated into the appropriate expression vector thus maintaining the open reading frame. Recombinant protein expression was undertaken according to the manufacturer's protocol. Subsequently, cells were harvested by centrifugation and the histidine-tagged proteins purified under denaturing conditions by Ni-NTA resin chromatography. Where required, purified proteins were dialysed extensively against decreasing concentrations of urea and finally against phosphate-buffered saline, pH 7·2.

Immunocytochemistry

Cercariae, lung-stage schistosomula and adult worms were prepared for immunocytochemistry based on the methodology developed by Riengrojpitak *et al*. (1989). Briefly, cercariae or cultured lung worms were pelleted in Eppendorf tubes by low-speed centrifugation. Ten freshly perfused and washed adult worms were hand-counted and pipetted into Eppendorf tubes in a small volume of RPMI-1640 medium (Gibco). Subsequently, parasites were mixed in optimum cutting temperature compound (OCT; Tissue-Tek, London, UK) on a cork disc and plunged into isopentane which had been pre-cooled in liquid nitrogen. Discs were then stored at −70 °C. All subsequent steps were performed as detailed previously (Riengrojpitak *et al*. 1989). Stained sections were examined under a Nikon fluorescence microscope and photographed on Kodak Ektachrome, ASA 400.

Assays of lymphocyte proliferation and cytokine production

Assays were undertaken as detailed previously (Mountford *et al*. 1995). Briefly, axillary lymph nodes were removed from 3 female C57Bl/6 mice 5 days after vaccination with 500 irradiated cercariae. Irradiated splenocytes were used as a source of antigen-presenting cells, at a concentration of 2×10^5/well. Lymph node cells were used at 10^5/well and stimulated with different antigens at a concentration of 0·5 μg/well. Soluble parasite preparations were used as positive controls and 2 pure non-schistosome proteins (BSA and β-galactosidase) served as negative controls. β-gal was expressed and purified in the pQe high level expression vector and therefore represents a good control for recombinant protein purity. Cells were also recovered by bronchoalveolar lavage from the lungs of mice 21 days after percutaneous exposure to irradiated cercariae as detailed by Smythies *et al*. (1992*b*).

Culture supernatants were removed at 72 h and stored at −80 °C for subsequent analysis of cytokine production which was undertaken using specific double-antibody ELISA as detailed previously (Mountford *et al*. 1995). Secretion of the type 1 and type 2 indicator cytokines, IFNγ and IL-4 respectively, was measured. No other cytokines were analysed in this study. Results illustrated are representative of 2 (lung) or 3 (lymph node) individual experiments. Where appropriate, the mean ± standard deviation has been plotted.

RESULTS

Western blotting

The antiserum raised against lung-stage released proteins was used to probe Western blots of SCAP, 0–3 h RPs, SLAP, 6–8 day RPs, and SWAP (Fig. 1). Between 5 and 20 proteins, ranging in molecular weight from 10 to 100 kDa were visible in the different antigen preparations. By far the most dominant band was one of approximately 50 kDa which was highly represented in SCAP and 0–3 h RPs, but barely visible in 6–8 day RPs. Most of the molecules detected in the lung-stage released proteins could also be found in the more complex soluble parasite preparations. However, there are

Table 1. Summary of the predicted identity of cDNA clones following homology searches of the GenBank and SwissProt databases

(The table details clones identified from cercarial, lung-stage schistosomula and adult worm cDNA libraries by screening with antiserum raised against lung worm released proteins.)

Clone identity	cDNA library	Number	Percentage
S. mansoni Fructose 1,6-*bis*phosphate aldolase	Cercarial	10	17·2
S. mansoni 21·7 kDa antigen	Cercarial	9	15·5
S. mansoni Calcium-binding protein	Cercarial	1	1·7
Unknowns	Cercarial	2	3·4
S. mansoni Fructose 1,6-*bis*phosphate aldolase	Lung	10	17·2
S. mansoni 21·7 kDa antigen	Lung	9	15·5
S. mansoni Paramyosin	Lung	3	5·2
S. mansoni Myosin	Lung	1	1·7
Unknowns	Lung	2	3·4
S. mansoni Paramyosin	Adult	4	6·9
S. mansoni Myosin	Adult	4	6·9
S. mansoni Calpain	Adult	2	3·4
Unknowns	Adult	1	1·7

obvious differences in the profile of proteins detected at the lung and adult worm stages, compared to those in SCAP and 0–3 h RPs, suggesting some stage-specific expression.

cDNA library screens

Having established that the anti-6–8 day RP serum was capable of detecting several antigens in a denatured form, cDNA library screening was undertaken. Approximately 3×10^6 pfu were sampled in the 3 different libraries; 22 positive clones were detected in the cercarial, 25 in the lung-stage and 11 in the adult worm library. After purification to homogeneity, all positive clones were sequenced, initially from the 5′ end. A summary of the clone identities following single-pass sequencing is given in Table 1.

Of the 11 different cDNAs identified, 6 had homology with previously sequenced *S. mansoni* genes, and 5 had no database matches. The most commonly identified molecules by library screening were the glycolytic enzyme fructose 1,6-*bis*phosphate aldolase, which alone accounted for more than one-third of all positive clones, and the 21·7 kDa antigen.

Full-length sequence analysis

Following single-pass sequence analysis, clones CL1, CL22, NLSL19, NLSL24 and A26 were found to have no homology to anything deposited in GenBank or SwissProt. Therefore, full-length sequence analysis was undertaken by primer walking from both 5′ and 3′ ends.

(*a*) *Clones CL1, CL22 and NLSL24* (*Accession numbers : AF030963, AF030964 and AF030962 respectively*). Clone CL1, identified from the cercarial cDNA library, contains an insert of 0·43 kBa and a single open reading frame of 92 amino acids which encodes a protein of predicted molecular weight 10·6 kDa. CL22, also identified from the cercarial library, contains an insert of 0·41 kBa and a small open reading frame of 52 amino acids which would code for a protein fragment of predicted molecular weight 6·5 kDa. Clone NLSL24, identified from the lung-stage library contains an insert of 0·42 kBa and encodes a protein of 119 amino acids which ends with an in-frame stop codon (UAA) at position 359. The predicted molecular weight of the protein is 13 kDa. Even when the complete cDNA sequence was known, searches of both GenBank and SwissProt databases showed no significant matches for any of these 3 clones.

(*b*) *NLSL19* (*Accession number : AF030960*). Clone NLSL19, identified from the lung-stage larval cDNA library, contained an insert of 1·45 kBa which was sequenced from both 5′ and 3′ ends. The clone was found to contain a single open reading frame of 427 amino acids which ends with an in-frame UAA stop codon at bp position 1282. The predicted molecular weight of the protein is 46·5 kDa with a pI of 4·65. The deduced amino acid sequence was found to have 51% identity over a 327 aa overlap with a human 26S proteasome subunit 5a (U51007) as well as significant yet lower homology with the *Drosophila melanogaster*, *Arabidopsis thaliana* and yeast 26S proteasome homologues. Figure 2 shows a multiple sequence alignment of clone NLSL19 with both human and *D. melanogaster* 26S proteasomes. It can be seen that the first 250 N-terminal amino acids and the extreme C-terminus are highly conserved between all 3 proteins. With the exception of an additional 19 amino acids starting at position 293 in the human proteasome and 33 amino acids in the *S. mansoni* homologue at position 360, few gaps are

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                        1                                                  50
D.melanogaster (p55035)  ......MVL ESTMISFDNS DFQRNGDYFP TRLIVQRDGI NLVCLTKLRS
Human (p55036)           .......MVL ESTMVCVDNS EYMRNGDFLP TRLQAQQDAV NIVCHSKTRS
Clone NLSL19             QVLIAYLMSQ EATIIAVDNS DYMRNGDFFP TRLQAQNDAV GLICQSKRQR
Consensus                -------M-- E-T----DNS ---RNGD--P TRL--Q-D-- ---C--K---

                        51                                                 100
D.melanogaster (p55035)  NPENNVGLMT LSNTVEVLAT LTSDAGRIFS KMHLVQPKGE INLLTGIRIA
Human (p55036)           NPENNVGLIT LANDCEVLTT LTPDTGRILS KLHTVQPKGK ITFCTGIRVA
Clone NLSL19             NPENTIGLLS LANT.EVLCT LTNDVSKIYN RLHLVEPKGR IIFCSSIRIA
Consensus                NPEN--GL-- L-N--EVL-T LT-D---I-- --H-V-PKG- I-----IR-A

                        101                                                150
D.melanogaster (p55035)  HLVLKHRQGK NHKMRIVVFV GSPINHEEGD LVKQAKRLKK EKVNVDIVSF
Human (p55036)           HLALKHRQGK NHKMRIIAFV GSPVEDNEKD LVKLAKRLKK EKVNVDIINF
Clone NLSL19             HLALRHRQLR HQKMRIVCFI GSPILEDEKE LTRLAKRLKK EKVNVDIINF
Consensus                HL-L-HRQ-- --KMRI--F- GSP----E-- L---AKRLKK EKVNVDI--F

                        151                                                200
D.melanogaster (p55035)  GDHGNNNEIL TAFINALNGK DGTGSHLVSV PRGSVLSDAL LSSPIIQGED
Human (p55036)           GEEEVNTEKL TAFVNTLNGK DGTGSHLVTV PPGPSLADAL ISSPILAGEG
Clone NLSL19             GENETNEQKL SEFIDTLNGK DGTGSHLISV APGTVLHDTL MTSPVVAGED
Consensus                G----N---L --F---LNGK DGTGSHL--V --G--L-D-L --SP---GE-

                        201                                                250
D.melanogaster (p55035)  GMGGAGLGGN VFEFGVDPNE DPELALALRV SMEEQRQRQE SEQRRANPDG
Human (p55036)           G.AMLGLGAS DFEFGVDPSA DPELALALRV SMEEQRQRQE EEARRAAAAS
Clone NLSL19             GSGMAG.AGL GLEFGLDGAE DPDLLYALRV SMEDQRMRQE HEVNGDGSNT
Consensus                G----G---- --EFG-D--- DP-L--ALRV SME-QR-RQE -E--------

                        251                                                300
D.melanogaster (p55035)  APPTGGDAGG GGGVSGSGPG NEESAGAENE ANTEEAMLQR ALALSTETPE
Human (p55036)           AAEAGIATTG TE........ .......... .DSDDALLKM TIS.QQEFGR
Clone NLSL19             SVVATSLPAG SG........ .......... .TSEEAMLQQ ALAMSMQMNN
Consensus                ---------G ---------- ---------- -----A-L-- ----------

                        301                                                350
D.melanogaster (p55035)  DN...LP... DFANMTEEEQ IAFAMQMSMQ DAP.DDSVTQ QAKRPKTDEA
Human (p55036)           TG...LP... DLSSMTEEEQ IAYAMQMSLQ GAEFGQAESA DIDASSAMDT
Clone NLSL19             TESSSLPMDI DLAAMSEEDQ IAYALRMSLQ QMGEETTQPT TTTLESDKTI
Consensus                -----LP--- D---M-EE-Q IA-A--MS-Q ---------- ----------

                        351                                                400
D.melanogaster (p55035)  NAPMDVDEDY S......... .......... .......... ...EVIGDPA
Human (p55036)           SEPAKEEDDY .......... .......... .......... ...DVMQDPE
Clone NLSL19             VEPSGVAMDI DQTPTKVTEN PNLSSSSGTL AAATSAVPTS ADLDVMYDAE
Consensus                --P-----D- ---------- ---------- ---------- ----V--D--

                        401                                              448
D.melanogaster (p55035)  FLQSVLENLP GVDPQSEAVR DAVGSLN.KD ..KDKKSDGK DSQKK...
Human (p55036)           FLQSVLENLP GVDPNNEAIR NAMGSLA.SQ ATKDGKKDKK EEDKK...
Clone NLSL19             FLESVLQSLP GVDTQNEDVR KAINALTKSQ SQRGSKKDEK EDEDKQNS
Consensus                FL-SVL--LP GVD---E--R -A---L---- -----K-D-K ----K---
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Fig. 2. Multiple sequence alignment of *Drosophila melanogaster* and human S5a 26S proteasome sequences and clone NLSL19. Identities are illustrated in the consensus line and gaps (.) introduced to optimize the alignment.

required for optimal alignment of the 3 sequences. The schistosome cDNA clone does not appear to have a start codon which adheres to the ANN.ATG.G motif commonly found in eukaryotes. However, from the alignment, it can be seen that the first methionine at position 22 lines up with the start codons of the human and *Drosophila* molecules. It is possible therefore that this may represent the start codon of the schistosome cDNA.

(*c*) *Clone A26* (*Accession number: AF030965*). Clone A26 contains an 800 bp insert with a single open reading frame of 171 amino acids which ends with an in-frame stop codon (UAA) at base pair position 514. From the deduced amino acid sequence, a protein of 19 kDa with a pI of 8·59 would be predicted. Homology searches at SwissProt showed small, yet significant similarities between the schistosome protein and a *D. melanogaster* lethal (1) discs large-1 tumour suppressor protein (P31007; 40% identity in 120 amino acid overlap) and a rat presynaptic density protein (P31016; 31% in 145 amino acid overlap). The homologies occur within an area known as disc homologous region 3 of both molecules. The deduced amino acid sequence contains a putative *N*-glycosylation site at amino acid residues 38–41 and an RGD motif exists at 110–112.

High-level expression

In total, 4 clones have been expressed and purified in the pQe high level expression vector; these include the previously cloned *S. mansoni* fructose 1,6-

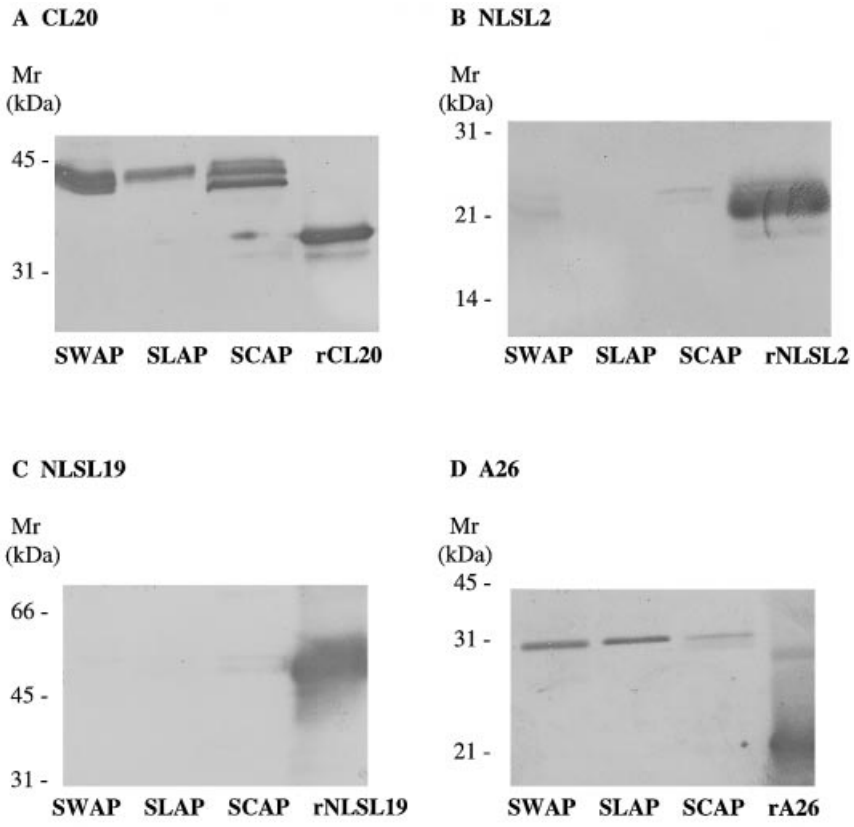

Fig. 3. Western blots of the target recombinant protein alongside SCAP, SLAP and SWAP probed with sera (1:100) against CL20 (A), NLSL2 (B), NLSL19 (C) and A26 (D). Samples of 5 μg soluble protein preparations and 0·5 μg recombinant protein were loaded per lane. Molecular weights are illustrated.

*bis*phosphate aldolase (CL20) and 21·7 kDa antigen (NLSL2) and the newly identified clones NLSL19 and A26. We were not able to express CL1, CL22 or NLSL24 in the pQe expression vector. The estimated molecular weights of each expressed recombinant protein were as follows: CL20, 35 kDa; NLSL2, 25 kDa; NLSL19, 56 kDa and A26, 19 kDa (data not shown). With the exception of clone NLSL19, the observed and expected molecular weights were very similar. The predicted molecular weight of NLSL19, however, is 46·5 kDa, a disparity of almost 10 kDa. The reason for this is not entirely clear.

Abundance and identification of 'native' protein in different life-cycle stages

The full-length target protein of each serum was identified by probing Western blots of SCAP, SLAP and SWAP (6–8 day RPs were not used for blotting because of the difficulty obtaining sufficient quantities). Figure 3 shows a panel of Western blots probed with serum against clones CL20, NLSL2, NLSL19 and A26. Antiserum against clone CL20 bound to the recombinant protein at 35 kDa, but in all 3 soluble preparations detected proteins at approximately 39 kDa (the predicted molecular weight of fructose 1,6-*bis*phosphate aldolase). In SCAP and SWAP, the antiserum recognized a doublet or possibly even a triplet of proteins of very similar molecular weight. Antisera against clones NLSL2 and NLSL19 bound strongly to their target recombinant proteins but only weakly to the full length molecule in any of the soluble preparations. Antiserum raised against clone A26 detected the 19 kDa target recombinant protein strongly and a protein of 30 kDa in all soluble parasite preparations.

Immunolocalization

Each serum was used to probe cryostat-cut sections of cercariae, lung-stage schistosomula or adult worms. Immunolocalization of each target protein is illustrated in Fig. 4. Clones CL20 (aldolase; Fig. 4A, B), NLSL2 (Sm21.7; Fig. 4E) and NLSL19 (proteasome homologue; Fig. 4C, D) all appeared to be most abundant in the muscle cells of the parasite. The anti-aldolase serum also bound to discrete foci of proteins within the cercaria, which could potentially be flame cells; in the adult worm the tegument was weakly positive. Antiserum against clone A26 (Fig. 4F) bound throughout all tissues but was strongest in the tegument.

Lymphocyte proliferation and cytokine production

The level of [^{3}H]thymidine incorporation by axillary lymph node cells, recovered from mice 5 days after

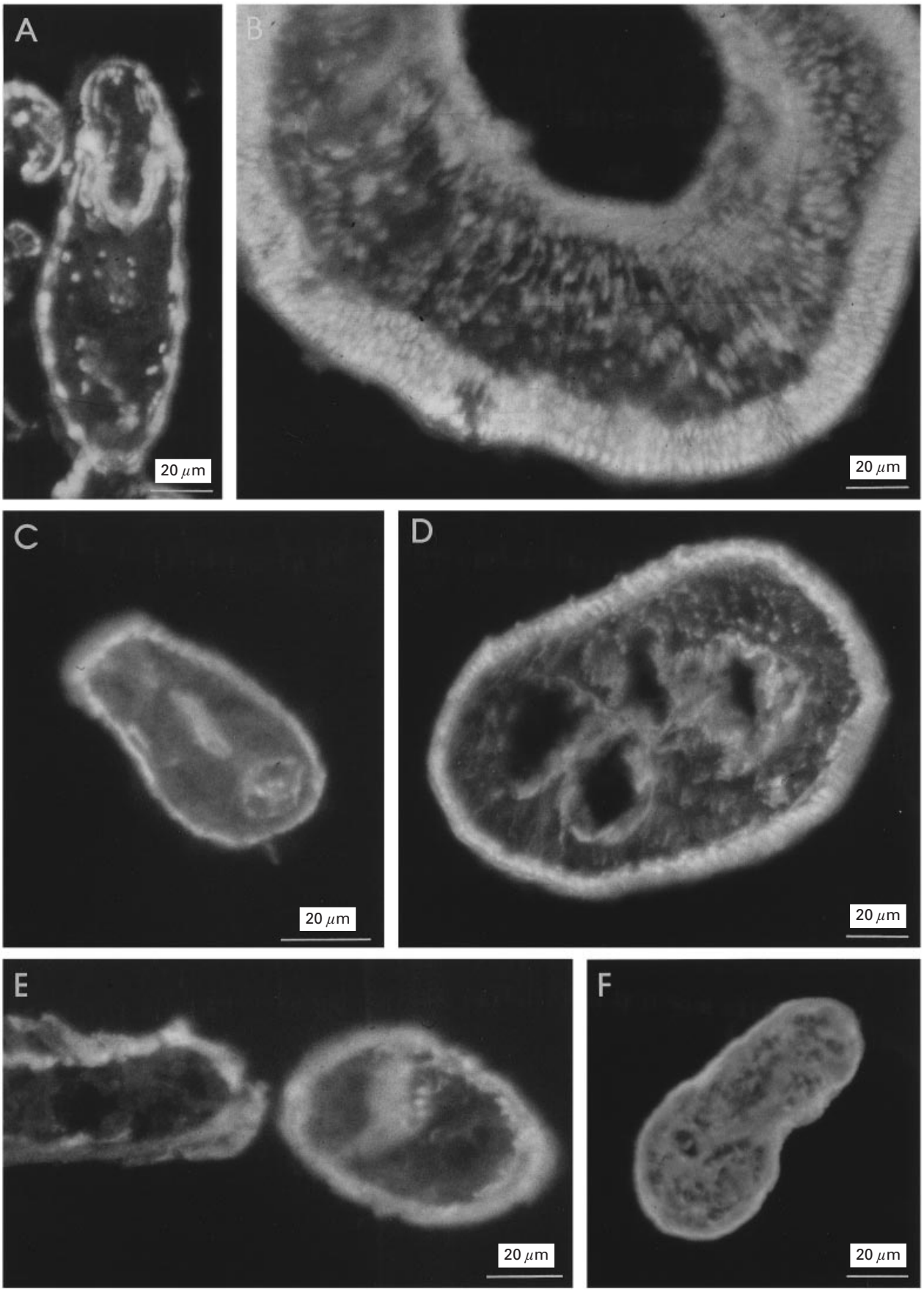

Fig. 4. Immunolocalization of clones CL20 (fructose 1,6-*bis*phosphate aldolase; (A, B), NLSL19 (C, D), NLSL2 (Sm21.7; E) and A26 (F) in sections of cercariae (A, E), lung-stage schistosomula (C, F) and adult worms (B, D).

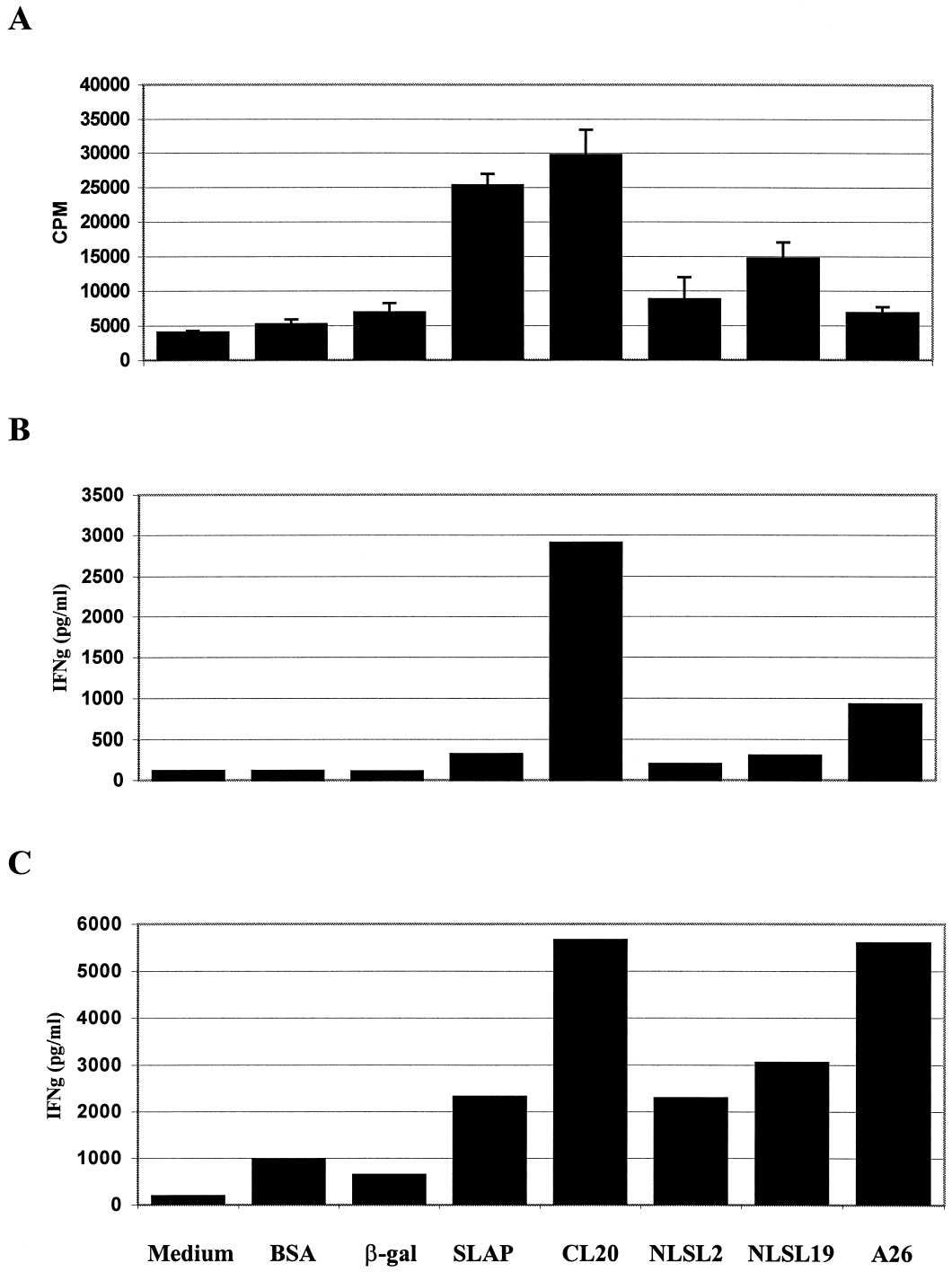

Fig. 5. Proliferative responses (A) and IFNγ production (B) and (C) by axillary lymph node cells recovered from mice 5 days after vaccination with irradiated cercariae (A and B) and from the lungs of mice 21 days post-vaccination (C) following stimulation with 0·5 μg antigen. Proliferative responses are expressed as counts per minute $\pm$ S.D. from triplicate wells. Representative results from 3 separate experiments are illustrated. IFNγ production is expressed as pg/ml following extrapolation from a standard curve.

vaccination, in response to different proteins is illustrated in Fig. 5A. Three of the 4 recombinants (NLSL2, NLSL19 and CL20) induced significantly higher levels of proliferation than the negative controls. The aldolase (CL20) caused greater cellular proliferation than the heterogeneous soluble preparation (SLAP) used as a positive control.

When cytokine production by lymph node cells was analysed, IL-4 was not detectable (data not shown). However, all recombinants induced greater levels of IFNγ secretion than the negative controls (Fig. 5B). In particular, when compared to SLAP, the aldolase induced more than 7 times the amount of IFNγ secretion. When cytokine production by cells recovered from the lungs of vaccinated mice was analysed, an even stronger response to A26 and

CL20 was observed with IFNγ production approaching 6000 pg/ml (Fig. 5C).

DISCUSSION

By probing Western blots with antiserum raised against lung-stage released proteins, we have identified molecules present in lung and other stages of the parasite life-cycle. Quantitatively, more molecules were detected in the 6–8 day RPs, against which the antiserum was raised, than in other fractions. However, the most dominant band recognized by this antiserum (50 kDa), was highly represented in both SCAP and 0–3 h RPs yet poorly represented in 6–8 day RPs and absent in other preparations, suggesting that it is highly immunogenic and expressed preferentially in earlier life-cycle stages. By screening cDNA libraries with antiserum raised against lung-stage released proteins, we have identified clones which encode both novel and previously characterized schistosome molecules. We believe this to be the first report of a strategy which specifically aimed to identify cDNA clones encoding antigens derived from lung worms. In total, 11 cDNAs encoding different proteins were isolated by library screening. Whether these represent molecules which are either excreted, secreted or released from the nephridia, gut or tegument is difficult to say. The two most frequently identified clones were found to encode the 39 kDa glycolytic enzyme fructose 1,6-*bis*phosphate aldolase and the Sm21.7 antigen. Together, these proteins accounted for 66% of all positive clones, but were only identified in the cercarial and lung-stage parasite cDNA libraries.

It is difficult to determine whether the aldolase and Sm21.7 are quantitatively the most common proteins found in the material released by lung worms, are relatively scarce but highly immunogenic and therefore yield serum with high aldolase and Sm21.7 antibody titres or, are simply very abundant in the 2 cDNA libraries. The fact that no dominant bands are seen at 39 kDa or 21·7 kDa following Western blotting with the 6–8 day RP antiserum supports the last hypothesis. In this respect, by using the lung-stage cDNA library as a source of expressed sequence tags, Franco *et al*. (1997) found that the aldolase accounted for 1·5% of all clones sampled whereas Sm21.7 was not identified. It is unclear why a glycolytic enzyme should be released by lung-stage schistosomula. However, other proteins involved in glycolysis such as PGK, TPI and G3PDH have been found on the surface of newly-transformed schistosomula (Lee *et al*. 1995; Harn *et al*. 1985; Goudot-Crozel *et al*. 1989, respectively) and therefore liable to release upon turnover of the tegument. The role of such molecules in this somewhat unusual location is not clear, although it has been postulated that they provide energy for repair of the tegument (Lee *et al*. 1995). Both the aldolase and Sm21.7 have been characterized previously (Doenhoff, 1998; Francis & Bickle, 1992, respectively), and their potential as vaccine candidates demonstrated. Stage-specific antigenic differences were noted for the aldolase, for which various explanations have been postulated; these included differential glycosylation, post-translational stage-dependent conformational changes and differential mRNA splicing. Such differences may account for the blotting pattern observed with the αCL20 serum.

Myosin and paramyosin were identified in both lung-stage and adult worm cDNA libraries. Since such molecules are associated with muscle, it would seem unlikely that they are released during membrane turnover by lung-stage larvae. However, there have been suggestions that paramyosin could be a secreted protein (Laclette *et al*. 1991; Nara *et al*. 1994). In addition, *S. mansoni* myosin and paramyosin are both vaccine candidates; therefore the immune system must be exposed to both molecules during the course of a challenge infection to enable subsequent immune-mediated parasite killing.

Clone NLSL19 was found to have significant sequence homology to the S5a subunit of the 26S proteasome of different species. The 26S proteasome is composed of 2 subcomplexes, the 20S proteasome (or multicatalytic protease) which contains the catalytic core of the protease, and the 19S regulatory complex. The 26S proteasome degrades ubiquitin-tagged proteins into small peptides and plays an important role in the selective degradation of abnormal/dysfunctional polypeptides. Subunit 5a of the proteasome, is thought to be the essential ubiquitin recognition component of the complex. cDNA clones encoding subunit 5a have been described previously in humans (Ferrel *et al*. 1996), *D. melanogaster* and *A. thaliana* (Van Nocker *et al*. 1996) and found to encode proteins of 40·3, 54 and 41 kDa respectively. The similar molecular weight, pI and significant sequence homology of NLSL19 to other known 26S proteasomes lead us to conclude that this clone encodes the schistosome homologue of such proteins. As far as we are aware, there has been no suggestion that such proteins are secreted and no signal peptide has been identified. In addition, immunolocalization of NLSL19 showed the protein to be associated primarily with muscle cells, therefore the process by which this protein is released is unclear.

Clone A26 shared some homology to 2 previously sequenced genes, although this did not provide insights into the molecule's function. The difference in molecular weight between the recombinant protein (19 kDa) and the molecule detected by the serum (30 kDa) suggests that the cDNA is incomplete. From the nucleotide sequence, it would

appear that the 5′ end is missing, therefore the identification of a putative leader sequence is impossible. Immunolocalization of A26 showed staining which was diffuse throughout many tissues, but strongest in the tegument. Although this does not provide compelling evidence that the protein is secreted or of membrane origin, the presence of *N*-glycosylation and cell attachment motifs (RGD) may be circumstantial evidence for a surface/secretory origin. RGD motifs are known to mediate many cell–cell and microbe–host interactions. In particular, the cellular matrix proteins vitronectin and fibronectin use this tripeptide to bind integrins present on cell surfaces. A number of viruses have been shown to use the RGD tripeptide to interact with cell receptors thus facilitating infection (Pulli, Koivunen & Hyypia, 1997). In addition, *Plasmodium falciparum* has also been found to contain the RGD sequence in the TRAP protein (Robson *et al*. 1988). This protein is expressed during the erythrocytic stage of the parasite life-cycle and may facilitate cellular invasion. Since schistosomes are extracellular parasites, we can only speculate what role, if any, this cell attachment motif is playing. It is possible that it could function as an intercellular cement. Alternatively, if exposed on the surface, the protein might provide a means by which host cells could adhere to the parasite thereby masking its presence from specific effector cells of the immune system.

Clones CL1, CL22 and NLSL24 showed no significant homology to any sequence deposited in GenBank to date and therefore likely represent novel genes. Since we were unable to express these clones in the pQe vector and thus raise antisera, it is impossible to speculate on the encoded protein's origin or function. One means of ascertaining whether a protein is secreted or transmembrane, is the presence of a signal peptide. The deduced amino acid sequence of each clone was submitted to an algorithm which predicts putative signal peptides (PSORT Prediction: http://psort.nibb.ac.jp). Clone CL1 appeared to have a cleavable signal peptide beginning at the second methionine. Analysis of the primary nucleotide sequence showed the flanking residues of the second ATG triplet to adhere to the ANN.ATG.G motif suggesting that this could be the start codon of the molecule. The presence of a signal peptide is known to be problematic in some high level expression systems and therefore may provide one explanation why we were unable to express this clone. The 5′ ends of clones NLSL24 and CL22 are lacking, indeed the deduced amino acid sequences contain no methionine residues, thus making it impossible to identify putative signal sequences Different high level expression vectors must be tested to try to find one suitable for the production of recombinant proteins from clones CL1, CL22 and NLSL24 and thus enable more detailed characterization.

The immunogenicity of the 4 expressed recombinant proteins was assessed by measuring cellular proliferation and production of the cytokines, IFNγ (type 1) and IL-4 (type 2). In this respect, we have demonstrated previously that the proteins secreted by lung-stage schistosomula are the most potent stimulators of IFNγ production by cells recovered from both lymph nodes and lungs of vaccinated mice. By cloning and expressing individual constituents of this heterogeneous subset of parasite proteins, we have been able to dissect the contribution that each makes to the immune response observed. Two of the 4 expressed recombinants elicit potent Th1-type immune responses by cells recovered from both lymph nodes and lungs of vaccinated mice. The quantity of IFNγ released in response to A26 and in particular to CL20 (aldolase) is very high and in both cases is greater than that caused by the soluble antigen preparation (SLAP) used as a positive control. Considering the number of antigens present in SLAP and therefore the number of T cell epitopes, this is perhaps surprising. Several explanations may exist. Since SLAP represents a complex mixture of proteins, it may contain molecules which promote or possibly down-regulate IFNγ production. Alternatively, it has been demonstrated previously that by day 21 post-vaccination, cells recovered by BAL from the lumen of mice are highly enriched with parasite-specific Th1 cells (Mountford *et al*. 1992; Coulson & Wilson, 1993). However, the extent of this schistosome-specific T cell repertoire is not known. It is possible that a large number of these cells are specific for a small number of epitopes. If such epitopes are contained in the recombinant proteins detailed here, a strong response might be predicted. Since immune elimination of challenge parasites in the irradiated cercaria vaccine model is dependent upon the secretion of IFNγ in the lungs, these results are promising in the search for new vaccine candidates.

We have employed a strategy which set out to identify proteins excreted, secreted or released by lung-stage schistosomula. Various criteria were applied to ascertain whether this goal had been achieved; these included sequence analysis of the identified clones, homology searches and immunolocalization of the encoded protein. It would appear that not all of the identified clones fulfill our initial criteria. Some encode proteins which are likely to be associated exclusively with muscle (myosin and paramyosin) or be cytosolic (calpain). One possible explanation for this finding is that some of the clones represent material leaked into the culture medium by the 1–5% of dead/dying parasites present in most cultures. Such proteins are likely to be abundant and immunogenic. Conversely, since schistosomes have evolved to survive in the potentially hostile environment of the host's bloodstream, those molecules secreted by schistosomula, unless being used as an

immunological smoke screen, are likely to be rare and poorly immunogenic (at least in the permissive human host). This would make them more difficult to characterize. However, in mice, immune responses to the 4 expressed recombinants were strong. In order to generate such a response, the immune system must be exposed to the target proteins. Since few if any attenuated parasites have died by day 5 (the time of lymph node removal) post-vaccination, this provides circumstantial evidence that the 4 proteins are truly released. Further work is required to determine the exact means by which the immune system is exposed to these proteins. However, it is hoped that this work provides further insight into the developmental biology of the parasite by identifying novel genes, but more importantly yields potential targets for chemotherapeutic and/or immunological intervention.

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